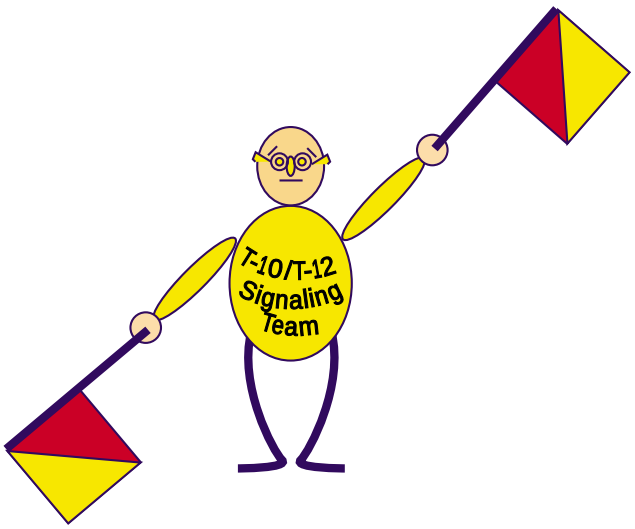
Instructions for the Kinetic Proofreading Programs[1]



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To request the programs, email redondo@lanl.gov specifying whether you want to run the programs on a PC or a Macintosh. The programs will be sent to you on a floppy disc. If you discover problems or have questions concerning the running of the programs, email redondo@lanl.gov.

1. Hlavacek, W., A. Redondo, H. Metzger, C. Wofsy and B. Goldstein. Kinetic proofreading models for cell signaling predict ways to escape kinetic proofreading. *Proc. Natl. Acad. Sci*. (in press).

Instructions for Kinetic Proofreading Programs
Up-dated February 26, 2001

1. Introduction to the Kinetic Proofreading Programs

In the context of cell signaling, "kinetic proofreading" refers to a mechanism for preventing the wrong molecules from eliciting cellular responses. If a signaling molecule must remain bound to a cellular receptor throughout a sequence of chemical reactions, in order to trigger a specific response, then other molecules that bind to the same receptor but dissociate rapidly will not stay bound long enough to trigger the response accidentally.

In many systems, signal transduction depends on receptor aggregation, induced by the signaling molecule. The Kinetic Proofreading programs 1.1, 2.1, and 3.1 simulate signaling pathways for the simplest aggregating receptor system, in which a bivalent ligand crosslinks pairs of monovalent receptors. The crosslinked "dimers" undergo a sequence of modifications (e.g., phosphorylation or association with an enzyme) leading to a state of the receptor or another molecule ("messenger") that can activate some cellular response (e.g., calcium influx or gene expression).

The programs generate ligand concentrations, and fractions of receptors in all possible states, at time points specified by the user. The user also specifies parameter values. The basic model is described in Section 2.1 and implemented in the program Kinetic Proofreading 1.1. Kinetic Proofreading 2.1 (Section 2.2) and 3.1 (Section 2.3) add a reaction scheme in which one dimer state activates a cytosolic messenger. In addition, Kinetic Proofreading 3.1 requires receptor dimers to associate with an initiating enzyme (e.g., a Src family kinase) to undergo modifications and activate messenger. All of the programs allow for down-regulation of receptors. Parameters are discussed in Section 3. The equations that constitute the models are given in Section 4.

2. Models and Programs

2.1. Kinetic Proofreading 1.1

The Model

The program kinetic_pr_1.1 (Kinetic Proofreading 1.1) reflects the model shown in Fig. 1. In the model, a symmetric bivalent ligand and a monovalent analogue bind to monovalent receptors. The only aggregate that can form is a dimer, i.e., a pair of receptors crosslinked by a bivalent ligand. Dimers are subject to a sequence of N reversible modifications. A newly formed dimer is in state 0 and the successive modified states are labeled 1 through N. The program allows for down-regulation of receptors by a mechanism that depends on aggregation (dimerization, in this model) and the extent of biochemical modification of a dimer. Dimers

in states numbered I or higher are subject to removal (e.g., by internalization, shedding, or sequestration). There must be at least one modification before a dimer can be internalized, i.e., $I > 0$. When aggregated receptors are removed from the available pool of receptors, we assume the ligand is not degraded and returns immediately to the pool of free ligands.

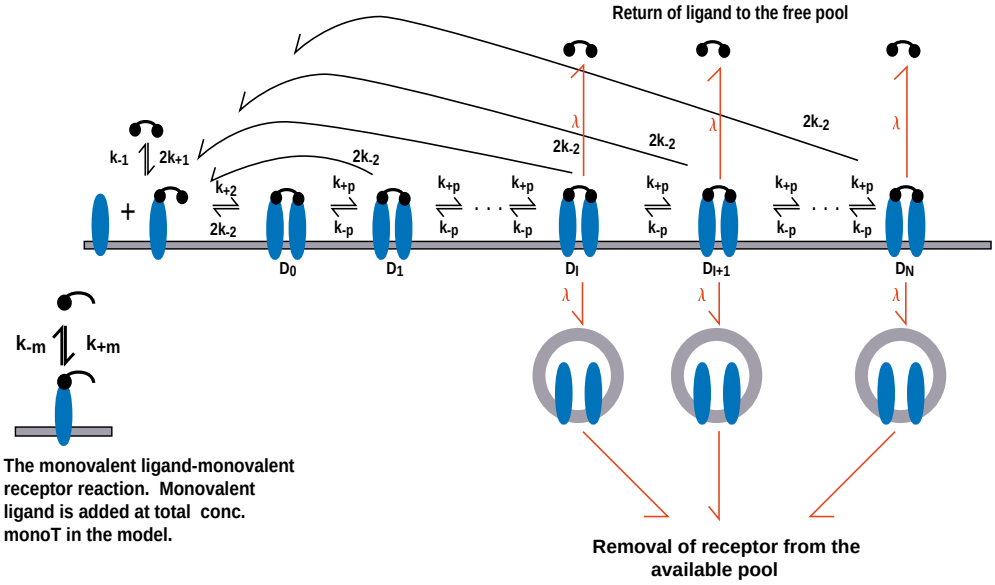

Fig. 1. Generalizing the kinetic proofreading model of McKeithan (1995, *Proc. Natl. Acad. Sci. USA.* 92:5042–5046) to include dimerization, reversible modifications, and down-regulation of receptors. We consider the case where a bivalent ligand interacts with monovalent receptors on the surface of a cell. We also allow for the addition of monovalent ligand.

The model assumes that signaling requires receptor aggregation. If a receptor dissociates from an aggregate, it immediately reverts to its original state. For example, if a receptor is phosphorylated in an aggregate, upon leaving the aggregate it immediately becomes dephosphorylated. k_{-2} is the dissociation rate constant for the opening of a bond between a site on a receptor in an aggregate and a site on a bivalent ligand. Since there are two bonds per aggregate (recall that only dimers can form in the model), the rate constant for a receptor leaving an aggregate is $2k_{-2}$.

Running the program kinetic_pr_1.1

Input for kinetic_pr_1.1

To run the program kinetic_pr_1.1, first edit the input file that contains parameter values. The parameter values will be discussed in Section 3. You may use any word-processing program. A typical input file, called binding.dat, is shown in Fig. 2. After you have edited the file, save it and *close it*.

```
1.0E+7    ! k+1 [1/(Ms)] forward rate constant for ligand-receptor binding
1.0E-2    ! k-1 [1/s] reverse rate constant
6.0E+6    ! rhoCell [cells/ml] cell density
3.0E+5    ! RT number of receptors per cell
1.0E-9    ! LT [M] bivalent ligand concentration
1.0E-0    ! k+2RTref [1/s] scaled rate constant for dimer formation
1.0E-2    ! k-2 [1/s] reverse rate constant
1.0E-1    ! k+p [1/s] forward rate constant for dimer modification
0.0E-1    ! k-p [1/s] reverse rate constant
1.2E-3    ! lambda [1/s] rate constant for receptor down-regulation
1.0E-6    ! monoT [M] concentration of added monovalent ligand
1.0E+7    ! k+m [1/(Ms)] forward rate constant for monovalent ligand-receptor binding
1.0E-2    ! k-m [1/s] reverse rate constant
```

Fig. 2. The input file for the program kinetic_pr_1.1.

Then start the executable program, kinetic_pr_1.exe, by opening it or clicking on the icon. You will be asked for additional information. A sample run is shown in Fig. 3.

```
Program Kinetic_PR, v. 1.1
 T-10/T-12 Cell Signaling Software
 Los Alamos National Laboratory
 Last modified 26 February 2001
 To integrate equations it uses subroutine RADAU5 by
 E. HAIRER AND G. WANNER, obtainable from
 http://www.unige.ch/math/folks/hairer/software.html

 Enter root of name for output files
sim
 Enter total number of modification steps:
3
 Enter number of first dimer subject to down-regulation (I>0):
4
 Enter time at which monovalent ligand is added:
4000
 Enter name of input file
binding.dat
 Enter maximum time in seconds
3600
 Enter time interval (in seconds) between data reports
10
```

Fig. 3. Input information.

The response to the first request for additional information (Fig. 3) determines the names of the output files. In the example, we choose the root "sim" and will have output files named sim1.out, sim2.out, and sim3.out.

Next, we choose to have three modification steps. There will be 4 possible dimer states, the unmodified dimer and three successively modified dimers. The user can choose any number of dimer states. If the total number of dimer states exceeds 10 (equivalently, if the number of modifications is greater than 9), additional output files will be created.

Then we specify that there is no down-regulation of receptors, by saying that the first dimer subject to removal from the pool of available receptors is dimer number 4, a number greater than the number of modifications.

Since monovalent ligand will not be added in this experiment, we specify that it will be added at 4000 s, i.e., beyond the maximum time point we will specify for the run (below).

The next request is for the name of the file with the input parameters, binding.dat in this case.

In entering the last two items for the sample run, we specify a total time of one hour (3600 s) and choose to print output at ten-second intervals.

Output from kinetic_pr_1

The first 100 s of the three output files are shown in Figs. 4 – 6. The columns of output are defined in the figure captions.

```
 Program Kinetic_PR, v. 1.1
 T-10/T-12 Cell Signaling Software
 Los Alamos National Laboratory
 Last modified 26 February 2001
 To integrate equations it uses subroutine RADAU5 by
 E. HAIRER AND G. WANNER, obtainable from
 http://www.unige.ch/math/folks/hairer/software.html

Total number of modification steps:                              3
Number of first dimer subject to down-regulation:                4
Maximum time in seconds:                                 3.600D+03
Time interval (in seconds) between data reports:         1.000D+01
Time at which monovalent ligand is added:                4.000D+03
Rates for bivalent ligand, k+1 & k-1 [1/(Ms), 1/s]:      1.000D+07   1.000D-02
Cell concentration [cells/ml], rhoCell:                  6.000D+06
Number of receptors per cell, RT:                        3.000D+05
Bivalent ligand concentration [M], LT:                   1.000D-09
Aggregation constants, k+2RTref & k-2 [1/s, 1/s]:        1.000D+00   1.000D-02
Dimer modification rates, k+p & k-p [1/s, 1/s]:          1.000D-01   0.000D-01
Down-regulation rate, lambda [1/s]:                      1.200D-03
Monovalent ligand concentration [M], monoT:              1.000D-06
Monovalent ligand-recept. rates, k+m & k-m [1/(Ms), 1/s]: 1.000D+07   1.000D-02

 time 2*D(i), i =  0  to   3
  0.000E-01  0.000E-01  0.000E-01  0.000E-01  0.000E-01
  1.000E+01  1.597E-01  6.914E-02  2.117E-02  6.100E-03
  2.000E+01  1.602E-01  1.163E-01  6.577E-02  4.566E-02
  3.000E+01  1.417E-01  1.218E-01  9.061E-02  1.105E-01
  4.000E+01  1.279E-01  1.141E-01  9.573E-02  1.760E-01
  5.000E+01  1.196E-01  1.057E-01  9.228E-02  2.296E-01
  6.000E+01  1.149E-01  9.976E-02  8.717E-02  2.692E-01
  7.000E+01  1.123E-01  9.598E-02  8.295E-02  2.973E-01
  8.000E+01  1.107E-01  9.370E-02  8.003E-02  3.171E-01
  9.000E+01  1.098E-01  9.234E-02  7.816E-02  3.313E-01
  1.000E+02  1.092E-01  9.152E-02  7.701E-02  3.415E-01
```

Fig. 4. The first output file (sim1.out for this example) includes the parameters and other information defining the simulated experiment. Then time (in seconds) and fractions of receptors in the distinct dimer states are printed out in successive columns.

```
time, rhoR, rhoL, rhoB, rhoInt, bMono, mono, Dim sum
  0.000E-01  1.000E+00  1.000E-09  0.000E-01  0.000E-01  0.000E-01  0.000E-01  0.000E-01
  1.000E+01  7.387E-01  6.003E-10  5.167E-03  0.000E-01  0.000E-01  0.000E-01  2.561E-01
  2.000E+01  6.073E-01  4.037E-10  4.801E-03  0.000E-01  0.000E-01  0.000E-01  3.879E-01
  3.000E+01  5.306E-01  2.887E-10  4.807E-03  0.000E-01  0.000E-01  0.000E-01  4.646E-01
  4.000E+01  4.813E-01  2.146E-10  4.943E-03  0.000E-01  0.000E-01  0.000E-01  5.137E-01
  5.000E+01  4.477E-01  1.638E-10  5.116E-03  0.000E-01  0.000E-01  0.000E-01  5.472E-01
  6.000E+01  4.237E-01  1.276E-10  5.289E-03  0.000E-01  0.000E-01  0.000E-01  5.711E-01
  7.000E+01  4.060E-01  1.009E-10  5.447E-03  0.000E-01  0.000E-01  0.000E-01  5.885E-01
  8.000E+01  3.928E-01  8.088E-11  5.585E-03  0.000E-01  0.000E-01  0.000E-01  6.016E-01
  9.000E+01  3.828E-01  6.561E-11  5.704E-03  0.000E-01  0.000E-01  0.000E-01  6.115E-01
  1.000E+02  3.750E-01  5.382E-11  5.803E-03  0.000E-01  0.000E-01  0.000E-01  6.192E-01
```

Fig. 5. The eight columns of the output file sim2.out give time (in seconds), rhoR = the fraction of receptors that are free, rhoL = the concentration of free bivalent ligand (in M), rhoB = the fraction of receptors that are bound to singly-bound bivalent ligand (i.e., the fraction of receptors that are bound to bivalent ligand but are not crosslinked to other receptors), rhoInt = the fraction of receptors that have been removed from the available pool by the down-regulation process (e.g., by internalization), bMono = the fraction of receptors that are bound to monovalent ligand, mono = the concentration of free monovalent ligand (in M), Dim sum = the sum of the fractions of receptors in the dimer states = the fraction of receptors in dimers.

```
time, R(i), i =  0  to   3
   0.000E-01  0.000E-01  0.000E-01  0.000E-01  0.000E-01
   1.000E+01  2.561E-01  9.641E-02  2.727E-02  6.100E-03
   2.000E+01  3.879E-01  2.278E-01  1.114E-01  4.566E-02
   3.000E+01  4.646E-01  3.229E-01  2.011E-01  1.105E-01
   4.000E+01  5.137E-01  3.859E-01  2.718E-01  1.760E-01
   5.000E+01  5.472E-01  4.276E-01  3.219E-01  2.296E-01
   6.000E+01  5.711E-01  4.561E-01  3.564E-01  2.692E-01
   7.000E+01  5.885E-01  4.762E-01  3.803E-01  2.973E-01
   8.000E+01  6.016E-01  4.909E-01  3.972E-01  3.171E-01
   9.000E+01  6.115E-01  5.018E-01  4.094E-01  3.313E-01
   1.000E+02  6.192E-01  5.100E-01  4.185E-01  3.415E-01
```

Fig. 6. The first column in sim3.out gives time in seconds and the second through last columns, denoted $R(i)$, $i = 0, \cdots, N$, give the cumulative fractions of receptors in dimer states i and above. In the example shown, the second column gives $R(0)$, the sum of fractions of receptors in all dimer states, modified and unmodified. The next column gives $R(1)$, the fraction of receptors in dimers with 1, 2, or 3 modifications. The following column gives $R(2)$, the fraction of receptors in dimers with 2 or 3 modifications. The last column gives $R(3)$, the fraction of receptors in dimers with all 3 of the possible modifications.

The program does not come with a plotting routine. The user must supply that. In Fig. 7a we plot the output from sim1.out. In Fig. 7b, for the same binding.dat file (Fig. 2), we plot the output when monovalent ligand is added at 150 s. The only change we made in going from **a** to **b** was that in **b**, when we entered the input information (Fig. 2), we entered 150 rather than 4000 for the time at which monovalent ligand is added.

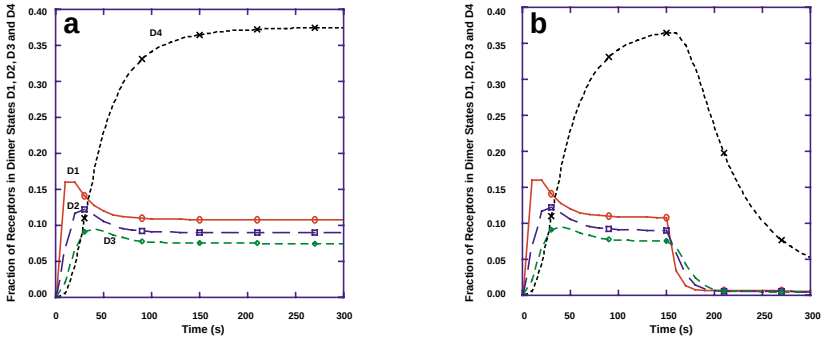

Fig. 7. Graphs of the fractions of receptors in the four dimer states, as functions of time, in simulated experiments where **a**. monovalent ligand is not added and **b**. monovalent ligand is added at 150 s. Plots are based on the output in sim1.out for the two cases. only the first 300 s are plotted.

2.2. Kinetic Proofreading 2

The Model

The program kinetic_pr_2.1 (Kinetic Proofreading 2.1) reflects the model shown in Fig. 1, with the additional reaction scheme shown in Fig. 8.

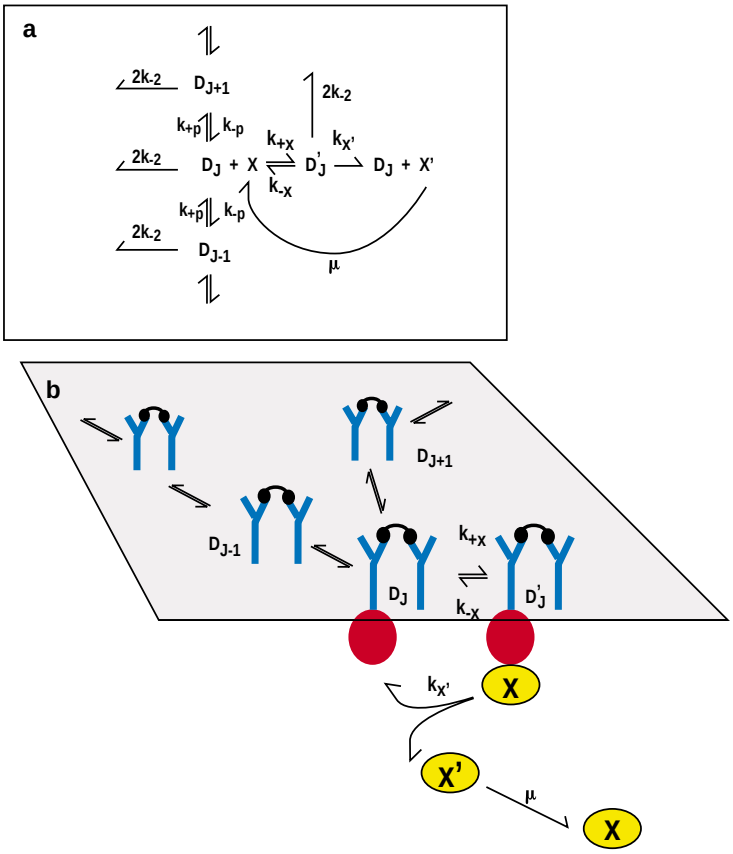

Fig. 8. The program kinetic_pr_2.1 allows the user to specify one dimer that mediates the activation or other modification of a soluble (cytosolic) "messenger." The dimer with J modifications (D_J, where $J > 1$) acts as an enzyme in the scheme shown in Fig. 8, the inactive messenger (X) as the substrate, and the active form of the messenger (X') as the product. The dimer and the inactive messenger combine reversibly to form a complex (D'_J), which then yields the activated messenger and the dimer. Notation for the reaction rate constants is indicated in Fig. 8. The active form of the messenger decays back to the inactive form with rate constant μ. In **a**, we show how the original model (Fig. 1) was modified to include the activation of a soluble messenger. In **b**, we present a depiction of the reaction scheme, omitting the reaction where dimers dissociate into two unaggregated receptors, one unbound and one bound to a site on a bivalent ligand. We only show the case where $I > J$, i.e., where the J^{th} dimer state is not internalized. The program also allows the J^{th} dimer to be internalized (when $I \leq J$). The program kinetic_pr_1.1 corresponds to the special case when there is no activation of a messenger. The program kinetic_pr_2.1

reduces to the program kinetic_pr_1.1 when either the total concentration of messenger, X_T, or the rate constant for complex formation, k_{+x}, is 0.)

Running the program kinetic_pr_2.1

Input for kinetic_pr_2.1

The input file differs from the input file for kinetic_pr_1.1 in that it contains values for five additional parameters, which characterize the reactions involving the cytosolic messenger.

```
1.0E+6    ! k+1 [1/(Ms)] forward rate constant for ligand-receptor binding
1.0E-2    ! k-1 [1/s] reverse rate constant
6.0E+6    ! rhoCell [cells/ml] cell density
5.0E0     ! rCell [microns] cell radius
3.0E+5    ! RT number of receptors per cell
1.0E-9    ! LT [M] bivalent ligand concentration
1.0E-0    ! k+2RTref [1/s] scaled rate constant for dimer formation
1.0E-2    ! k-2 [1/s] reverse rate constant
1.0E-1    ! k+p [1/s] forward rate constant for dimer modification
0.0E-1    ! k-p [1/s] reverse rate constant
1.2E-3    ! lambda [1/s] rate constant for receptor down-regulation
1.0E-6    ! monoT [M] concentration of added monovalent ligand
1.0E+6    ! k+m [1/(Ms)] forward rate constant for monovalent ligand-receptor binding
1.0E-2    ! k-m [1/s] reverse rate constant
5.0E+5    ! k+x [1/(Ms)] forward rate constant for messenger-dimer binding
1.0E-2    ! k-x [1/s] reverse rate constant
1.0E-1    ! kxprime [1/s] rate constant for messenger activation
5.0E-3    ! mu [1/s] rate constant for messenger decay
1.0E+5    ! XT number of messenger molecules per cell
```

Fig. 9. The input file for the program kinetic_pr_2.1.

To run the executable program kinetic_pr_2.exe, the user enters the same information as for kinetic_pr_1.exe, and is also asked for the number of the dimer state that mediates activation of the messenger. In the sample run shown in Fig. 10, we specify that the dimer with two modifications is the one that activates the messenger, i.e., $J = 2$.

```
 Program Kinetic_PR v. 2.1
 T-10/T-12 Cell Signaling Software
 Los Alamos National Laboratory
 Last modified 26 February 2001
 To integrate equations it uses subroutine RADAU5 by
 E. HAIRER AND G. WANNER, obtainable from
 http://www.unige.ch/math/folks/hairer/software.html

 Enter root of name for output files
sim
 Enter total number of modification steps:
3
 Enter number of first dimer subject to down-regulation (I>0):
4
 Enter number J (>1) of dimer that binds messenger:
2
 Enter time at which monovalent ligand is added:
4000
 Enter name of input file
binding.dat
 Enter maximum time in seconds
3600
 Enter time interval (in seconds) between data reports
10
```

Fig. 10. Input information for kinetic_pr_2.exe.

Output from kinetic_pr_2.1

There is one more output file than for kinetic_pr_1.1. In the example, the file is sim3.out. The first column gives the time in seconds. The other columns give fractions of messenger in the inactivated state, the activated state, and the complex with the activating dimer.

```
time, x, x prime, x-complex
   0.000E-01  1.000E+00  0.000E-01  0.000E-01
   1.000E+01  9.945E-01  1.134E-03  4.337E-03
   2.000E+01  9.647E-01  1.313E-02  2.217E-02
   3.000E+01  9.062E-01  4.630E-02  4.755E-02
   4.000E+01  8.223E-01  1.036E-01  7.412E-02
   5.000E+01  7.183E-01  1.831E-01  9.864E-02
   6.000E+01  5.995E-01  2.810E-01  1.195E-01
   7.000E+01  4.721E-01  3.922E-01  1.356E-01
   8.000E+01  3.438E-01  5.108E-01  1.454E-01
   9.000E+01  2.248E-01  6.290E-01  1.462E-01
   1.000E+02  1.291E-01  7.364E-01  1.346E-01
```

Fig. 11 The output file sim3.out prints time (in seconds) and fractions of messenger in the inactive state (notation x), the active state (x prime), and the complex with the dimer that mediates the transition (complex).

In Fig. 12a we plot the output from sim3.out for the first 2000 s. In Fig. 12b, for the same binding.dat file, we plot the output when monovalent ligand is added at 1000 s. The only change we made in going from **a** to **b** was that in **b**, when we entered the input information (Fig. 10), we entered 1000 rather than 4000 for the time at which monovalent ligand is added.

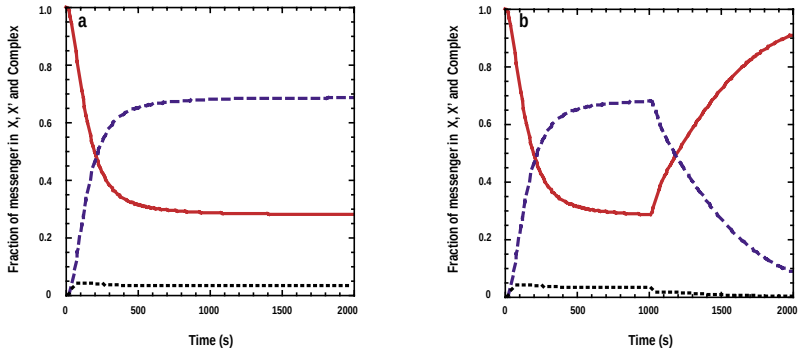

Fig. 12. Plot of the time course of the fraction of messenger in the inactive form (x), the active form (x'), and the complex the messenger forms with the dimer that mediates the transition (x-complex), **a**. without adding monovalent ligand and **b**. adding monovalent ligand at 600 s. Plots are based on the output in sim3.out for the two cases.

2.3. Kinetic Proofreading 3.1

The Model

The program kinetic_pr_3.1 (Kinetic Proofreading 3.1) adds another step to the kinetic proofreading model. It assumes that a surface-associated enzyme such as an initiating kinase must

associate with a receptor aggregate before any further modifications of the receptors can occur. The additional reaction is shown in Fig. 13. In this model, when internalization occurs, the enzyme is not degraded but is immediately returned to the pool of free enzyme.

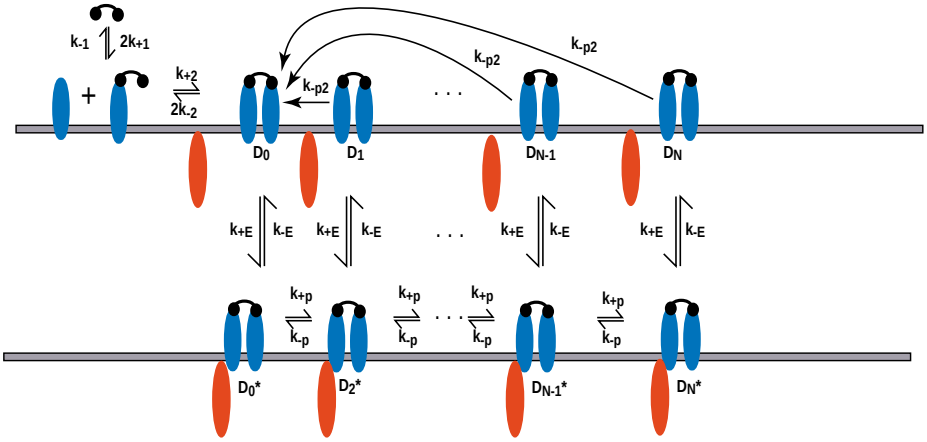

Fig. 13. The Kinetic Proofreading 3.1 model requires that an aggregate must associate with a surface-associated enzyme before any biochemical modifications can occur. Association with the enzyme is a reversible reaction. When the enzyme is present, dimers undergo reversible modifications with forward and reverse rate constants k_{+p} and k_{-p}. If the enzyme dissociates from a modified dimer, the modifications are reversed rapidly; the dimer reverts to the unmodified state with rate constant k_{-p2}. For simplicity, two reactions are omitted from the figure but not from the model. As in the previous models, dimers can break up; then all modifications, including association with the enzyme, are immediately reversed. Also, receptors in dimer states numbered I and above, with or without associated enzyme, are subject to down regulation. When dimers are lost, associated enzyme is not down-regulated but returns to the pool of free enzyme.

In the special case when the initiating enzyme is in large excess, all dimers are associated with an initiating enzyme and the program kinetic_pr_3 reduces to kinetic_pr_2. In this limit, the fraction of receptors in the i^{th} dimer-enzyme complex, as predicted by kinetic_pr_3.1, is the same as the fraction predicted by kinetic_pr_2.1 for the i^{th} dimer state.

Running the program kinetic_pr_3.1

Input for kinetic_pr_3.1

The input file for kinetic_pr_3.1 expands the input file for kinetic_pr_2.1 by four additional entries, three that characterize the interaction of dimers with the enzyme (ET, k+EETref,k-E) and k-p2, the rate constant for a modified dimer that is no longer associated with the

initiating enzyme to revert to its unmodified form. These additional parameters are defined, along with the other parameters, in Section 3.

```
1.0E+6    ! k+1 [1/(Ms)] forward rate constant for ligand-receptor binding
1.0E-2    ! k-1 [1/s] reverse rate constant
6.0E+6    ! rhoCell [cells/ml] cell density
5.0E-0    ! rCell [microns] cell radius
3.0E+5    ! RT number of receptors per cell
1.0E-9    ! LT [M] bivalent ligand concentration
1.0E-0    ! k+2RTref [1/s] scaled rate constant for dimer formation
1.0E-2    ! k-2 [1/s] reverse rate constant
1.0E-1    ! k+p [1/s] forward rate constant for dimer modification
0.0E-1    ! k-p [1/s] reverse rate constant
5.0E-2    ! k-p2 [1/s] deactivation rate constant for modified dimers without enzyme
1.2E-3    ! lambda [1/s] rate constant for receptor down-regulation
1.0E-6    ! monoT [M] concentration of added monovalent ligand
1.0E+6    ! k+m [1/(Ms)] forward rate constant for monovalent ligand-receptor binding
1.0E-2    ! k-m [1/s] reverse rate constant
5.0E+5    ! k+x [1/(Ms)] forward rate constant for messenger-dimer binding
1.0E-2    ! k-x [1/s] reverse rate constant
1.0E-1    ! kxprime [1/s] rate constant for messenger activation
5.0E-3    ! mu [1/s] rate constant for messenger decay
1.0E+5    ! XT number of messenger molecules per cell
3.0E+4    ! ET number of initiating enzyme molecules per cell
1.0E+1    ! k+EETref [1/s] scaled rate constant for initiating enzyme-receptor
1.0E-1    ! k-E [1/s] reverse rate constant
```

Fig. 14. The input file for the program kinetic_pr_3.1.

The executable program kinetic_pr_3.exe asks the user to enter exactly the same information as for kinetic_pr_2.exe.

Output from kinetic_pr_3.1

Fractions of receptors in dimers, with and without associated enzyme, are given in distinct output files. Figure 15 shows an output file, sim2.out, that lists fractions of receptors in dimers associated with enzyme. For this example, the input was the same as in Fig. 10. Another file, sim1.out, lists fractions of receptors in the four dimer states without enzyme. Enzyme-associated dimers are indicated by a star notation.

```
time 2*Dstar(i), i =  0  to   3
   0.000E-01  0.000E-01  0.000E-01  0.000E-01  0.000E-01
   1.000E+01  2.254E-02  8.195E-03  1.721E-03  4.597E-04
   2.000E+01  3.239E-02  1.879E-02  6.365E-03  3.839E-03
   3.000E+01  3.741E-02  2.552E-02  1.098E-02  1.051E-02
   4.000E+01  4.079E-02  2.950E-02  1.466E-02  1.903E-02
   5.000E+01  4.348E-02  3.197E-02  1.736E-02  2.782E-02
   6.000E+01  4.580E-02  3.359E-02  1.921E-02  3.564E-02
   7.000E+01  4.791E-02  3.470E-02  2.039E-02  4.173E-02
   8.000E+01  4.995E-02  3.555E-02  2.111E-02  4.584E-02
   9.000E+01  5.200E-02  3.631E-02  2.156E-02  4.818E-02
   1.000E+02  5.405E-02  3.709E-02  2.189E-02  4.919E-02
```

Fig. 15. olumn 1 gives time in seconds. Columns 2 – 5 give fractions of receptors in the four dimer states associated with the initiating enzyme.

The file that lists various ligand and receptor concentrations (analogous to Fig. 5 for kinetic_pr_1.1) contains an additional column, giving the concentration of free enzyme (Fig. 16).

```
time, rhoR, rhoL, rhoInt, rhoB, bMono, mono, Dim sum, rhoEnz
   0.000E-01  1.000E+00  1.000E-09  0.000E-01  0.000E-01  0.000E-01  0.000E-01  0.000E-01  1.000E+00
   1.000E+01  9.628E-01  9.430E-10  0.000E-01  7.492E-04  0.000E-01  0.000E-01  3.649E-02  8.324E-01
   2.000E+01  9.283E-01  8.913E-10  0.000E-01  8.421E-04  0.000E-01  0.000E-01  7.081E-02  6.728E-01
   3.000E+01  8.970E-01  8.440E-10  0.000E-01  9.352E-04  0.000E-01  0.000E-01  1.021E-01  5.327E-01
   4.000E+01  8.682E-01  8.008E-10  0.000E-01  1.028E-03  0.000E-01  0.000E-01  1.307E-01  4.122E-01
   5.000E+01  8.418E-01  7.611E-10  0.000E-01  1.121E-03  0.000E-01  0.000E-01  1.570E-01  3.124E-01
   6.000E+01  8.175E-01  7.245E-10  0.000E-01  1.213E-03  0.000E-01  0.000E-01  1.813E-01  2.341E-01
   7.000E+01  7.951E-01  6.906E-10  0.000E-01  1.305E-03  0.000E-01  0.000E-01  2.036E-01  1.769E-01
   8.000E+01  7.742E-01  6.593E-10  0.000E-01  1.396E-03  0.000E-01  0.000E-01  2.244E-01  1.373E-01
   9.000E+01  7.549E-01  6.301E-10  0.000E-01  1.485E-03  0.000E-01  0.000E-01  2.436E-01  1.105E-01
   1.000E+02  7.369E-01  6.030E-10  0.000E-01  1.574E-03  0.000E-01  0.000E-01  2.615E-01  9.213E-02
```

Fig. 16. Output file sim3.out for kinetic_pr_3.1.

3. Parameters

The reader is referred to the model in Fig. 1 and the file of input parameters, Fig. 2. k_{+1} and k_{-1} are the forward and reverse rate constants for the binding of a site on a bivalent ligand in solution to a receptor site. The association and dissociation rate constants for monovalent ligand are k_{+m} and k_{-m}. The forward rate constants k_{+1} and k_{+m} must be entered in M^{-1} s^{-1}. The reverse rate constants k_{-1} and k_{-m} are in s^{-1}.

rhoCell is the concentration of cells, in cells/ml. This parameter is used in the programs to convert receptor concentrations to molar units, to deal appropriately with receptor-ligand binding.

RT is the total concentration of receptors, in number per cell.

LT is the total concentration of bivalent ligand and monoT is the total concentration of monovalent ligand. The ligand concentrations are in molar units (M).

k_{-2} is the single site dissociation rate constant for a doubly bound ligand. It is reasonable to assume that $k_{-1} = k_{-2}$, although if there is additional strain introduced on the bonds when the second bond forms, then we would expect that $k_{-2} > k_{-1}$.

k_{+2} is the forward rate constant for dimer formation. The equilibrium aggregation constant is $K_2 = k_{+2}/k_{-2}$. Note that k_{+2} describes a surface reaction, i.e. a reaction between a receptor-ligand complex and a free receptor. Its units are a surface concentration per second, for example cm^2/s. If $\tilde{R}T$ is the receptor concentration in receptors/cm^2, then $k_{+2}\tilde{R}T$ has units of s^{-1}. Rather than entering k_{+2}, we enter the quantity k+2RTref=$k_{+2}\tilde{R}T$, in units of s^{-1}, where $\tilde{R}T$ is the surface concentration of receptors on a "reference cell" with 100,000 receptors per cell. It is important to realize that at equilibrium, if $K_2\tilde{R}T \ll 1$, there will be little dimer formation. In the example we have given, Fig. 2, $K_2\tilde{R}T$ =(k+2RTref/k_{-2})$\times$ (RT/10^5)=300. If in binding.dat we gave RT as 1×10^3 instead of 3×10^5 but kept k+2RTref and k_{-2} the same, then the program would calculate $K_2\tilde{R}T = 1.0$ and there would be considerably less dimer formation.

k_{+p} and k_{-p} are the forward and reverse rate constants for the modification reactions and are entered in s^{-1}.

The reader is now referred to Figs. 8 and 9, for the additional parameters needed in the programs kinetic_pr_2.1 and kinetic_pr_3.1 to describe the activation of an intracellular messenger. In principal, a messenger could be cytosolic or membrane-associated, but the programs deal only with the case where the messenger is a cytosolic molecule.

k_{+x} and k_{-x} are the forward and reverse rate constants for the association of the soluble messenger with the J^{th} dimer. These rate constants have units of M^{-1} s^{-1} and s^{-1} respectively. kx prime=$k_{x'}$ (given in s^{-1}) is the forward rate constant for the activation of the soluble messenger, after the inactive messenger has formed a complex with the activating dimer. mu=μ, in s^{-1}, is the rate constant for decay of active messenger to the inactive form. It is a critical parameter in determining whether or not the addition of monovalent ligand will cause the rapid loss of activated messenger.

XT is the total number of messenger molecules in the cell. In the example, Fig. 9, XT=100,000 and there is one soluble messenger for every three receptors. By entering XT in this way, it is easy to look at the limits where the messenger is in excess or is limiting in comparison with receptors.

rCell is the radius of the cell, in microns. In the program, XT is converted to a solution concentration (M) by taking the volume of the cell to be $4\pi(\text{rCell})^3/3$. (The cell radius, rCell, also affects surface reactions – dimerization and, in kinetic_proofreading_3.1, association with enzyme – because surface concentrations depend on surface area, but we have not used rCell explicitly in this context. The values of k+2RTref and k+EETref (see below) entered or determined in a set of experiments reflect rCell implicitly.)

There are four additional parameters in kinetic_proofreading_3.1 (Figs. 13 and 14), three characterizing the interaction, at the cytoplasmic face of the cell membrane, between receptor dimers and a membrane-associated initiating enzyme, and one characterizing the reversion of a non-enzyme-associated dimer to the unmodified form.

ET is the total number of molecules per cell of the initiating enzyme, which we assume to be surface associated.

k+EETref=$k_{+E}\tilde{E}T$, entered in units of s^{-1}, is the product of k_{+E}, the forward rate constant for the association of the initiating enzyme with a receptor dimer, and $\tilde{E}T$, the surface concentration of the enzyme in a reference cell containing 100,000 membrane-associated enzyme molecules.

k_{-E}, in units of s^{-1}, is the rate constant for dissociation of enzyme from a dimer.

k_{-p2}, in units of s^{-1}, is the rate constant for a modified dimer that is not associated with an initiating enzyme to revert to an unmodified dimer.

Appendix: Equations for the Kinetic Proofreading Models

In the kinetic proofreading programs, the equations give the number of receptors or dimers in a given state, relative to the total number of receptors. This form of the equations for kinetic_pr_1, in terms of nondimensional "concentrations," is in Section A.2. We start in Section A.1 with the dimensional equations on which the nondimensionalization is based. The later sections show how the systems of equations must be extended to deal with messenger and initiating enzyme in kinetic_pr_2 and kinetic_pr_3.

A.1. Dimensional form of equations for kinetic_pr_1

A bivalent ligand (total concentration L_T, free concentration L, units M) and a monovalent analogue (total concentration L'_T, free concentration L', units M) bind to monovalent receptors. Receptor dimers form and undergo a sequence of reversible modifications. The states and rate constants are summarized in Fig. 1.

There must be at least one modification before a dimer is subject to the aggregation-mediated down-regulation process. Dimers in states $i \geq I$ (where $I \geq 2$) are removed, with rate constant λ (s^{-1}), from the pool of receptors that are available to interact with ligand. In Fig. 1, removal (down-regulation) is pictured as internalization.

The following concentrations of receptors and dimers are in number per cell.

R_T = total receptor concentration
R = free receptor concentration
B = concentration of isolated (non-crosslinked) receptors, bound to bivalent ligand
= concentration of singly bound bivalent ligand, in number per cell
B' = concentration of receptors bound to monovalent ligand
= concentration of bound monovalent ligand, in number per cell
D_i = concentration of dimers in state i $\quad (i = 1, \cdots, N)$
$D = \sum_{i=1}^{N} D_i$
R_I = number of receptors per cell that have been removed in the down regulation process

Rate constants for binding and dissociation of monovalent ligand are k_{+m} (M^{-1} s^{-1}) and k_{-m} (s^{-1}). Single site rate constants for binding and dissociation of bivalent ligand are k_{+1} (M^{-1} s^{-1}) and k_{-1} (s^{-1})

When concentrations of receptors are given in number per cell, then the forward rate constant for dimer formation, k_{+2}, must be given in s^{-1}. In Section 3 we noted that if receptor concentrations are given in receptors/cm^2, then k_{+2} has units of cm^2/s. For both sets of units, the product $k_{+2}R_T$ (in s^{-1}) has the same value. It is the product that must be specified in the file of input parameter values (Fig. 2). In writing the equations, it is convenient to start with receptor concentrations in numbers per cell, to deal consistently with the pool of internalized receptors.

The single site dissociation constant for a bivalent ligand bound to two receptors is k_{-2} (s^{-1}).

The forward and reverse rate constants for the successive modifications of dimers are k_{+p} (s^{-1}) and k_{-p} (s^{-1}).

The receptor concentrations satisfy the following system of ordinary differential equations

$$\begin{aligned}
\frac{dB}{dt} &= 2k_{+1}LR - k_{-1}B - k_{+2}BR + 2k_{-2}D \\
\frac{dB'}{dt} &= k_{+m}L'R - k_{-m}B' \\
\frac{dD_1}{dt} &= k_{+2}BR - (2k_{-2} + k_{+p})D_1 + k_{-p}D_2 \\
\frac{dD_i}{dt} &= k_{+p}D_{i-1} - (2k_{-2} + k_{+p} + k_{-p})D_i + k_{-p}D_{i+1} \qquad 2 \le i \le \min\{I-1, N-1\} \\
\frac{dD_i}{dt} &= k_{+p}D_{i-1} - (2k_{-2} + k_{+p} + k_{-p} + \lambda)D_i + k_{-p}D_{i+1} \qquad I \le i \le N-1 \\
\frac{dD_N}{dt} &= \begin{cases} k_{+p}D_{N-1} - (2k_{-2} + k_{-p} + \lambda)D_N & \text{if } I \le N \\ k_{+p}D_{N-1} - (2k_{-2} + k_{-p})D_N & \text{if } I > N \end{cases} \\
\frac{dR_I}{dt} &= 2\lambda \sum_{i=I}^{N} D_i
\end{aligned}$$

with initial conditions

$$\begin{aligned}
&B(0) = 0 \\
&B'(0) = 0 \\
&D_i(0) = 0 \qquad 1 \le i \le N \\
&R_I(0) = 0
\end{aligned}$$

and conservation laws

$$\begin{aligned}
R_T &= R + B + B' + 2D + R_I \\
L_T &= L + \text{factor}_1(B + D)/R_T \\
L'_T &= L' + \text{factor}_1 B'/R_T
\end{aligned}$$

where

$$\text{factor}_1 = R_T\rho/(6 \times 10^{20}) \text{ M}$$

A.2. Equations with receptor concentrations in nondimensional form

Renormalizing the variables as follows: $r = R/R_T$, $b = B/R_T$, $b' = B'/R_T$, $d_i = D_i/R_T$, $d = D/R_T$, $r_I = R_I/R_T$, and taking $\tilde{k}_{+2} = k_{+2}R_T$ the equations become:

$$\frac{db}{dt} = 2k_{+1}Lr - k_{-1}b - \tilde{k}_{+2}br + 2k_{-2}d$$
$$\frac{db'}{dt} = k_{+m}L'r - k_{-m}b'$$
$$\frac{dd_1}{dt} = \tilde{k}_{+2}br - (2k_{-2} + k_{+p})d_1 + k_{-p}d_2$$
$$\frac{dd_i}{dt} = k_{+p}d_{i-1} - (2k_{-2} + k_{+p} + k_{-p})d_i + k_{-p}d_{i+1} \qquad 2 \le i \le \min\{I-1, N-1\}$$
$$\frac{dd_i}{dt} = k_{+p}d_{i-1} - (2k_{-2} + k_{+p} + k_{-p} + \lambda)d_i + k_{-p}d_{i+1} \qquad I \le i \le N-1$$
$$\frac{dd_N}{dt} = \begin{cases} k_{+p}d_{N-1} - (2k_{-2} + k_{-p} + \lambda)d_N & \text{if } I \le N \\ k_{+p}d_{N-1} - (2k_{-2} + k_{-p})d_N & \text{if } I > N \end{cases}$$
$$\frac{dr_I}{dt} = 2\lambda \sum_{i=I}^{N} d_i$$

with initial conditions and conservation laws

$$b(0) = 0$$
$$b'(0) = 0$$
$$d_i(0) = 0 \qquad 1 \le i \le N$$
$$r_I(0) = 0$$
$$1 = r + b + b' + 2d + r_I$$
$$L_T = L + \text{factor}_1(b + d)$$
$$L'_T = L' + \text{factor}_1 b'$$

A.3. Activation of cytosolic messenger

In the program kinetic_pr_2, we allow one of the modified dimer states (the J^{th}, where $J \ge 2$) to mediate the transition of a soluble cytosplasmic messenger with concentration X, to an active form with concentration X', according to the scheme shown in Fig. 8. The concentrations are in number per cell. The dimer and messenger interact reversibly with forward and reverse rate constants k_{+x} (M^{-1} s^{-1}) and k_{-x} (s^{-1}) to form a complex with concentration D'_J. With rate constant $k_{x'}$ (s^{-1}), the complex yields the activated form of the messenger and the dimer. Activated messenger decays back to the inactive form with rate constant μ (s^{-1}). Then the following equations are added to the dimensional equations in Section 4.1

$$\frac{dX}{dt} = -k_{+x}\tilde{X}D_J + (k_{-x} + 2k_{-2})D'_J + \mu X'$$
$$\frac{dX'}{dt} = k_{x'}D'_J - \mu X'$$
$$\frac{dD'_J}{dt} = k_{+x}\tilde{X}D_J - (k_{-x} + k_{x'} + 2k_{-2})D'_J$$

where $\tilde{X}$ is the solution concentration of free messenger in M. The equation for D_J is modified by adding $-k_{+x}\tilde{X}D_J + (k_{-x} + k_{x'})D'_J$. The definition of the receptor dimer concentration is extended to include D'_J, i.e.,

$$D = \sum_{i=1}^{N} D_i + D'_J$$

The conservation law for the soluble messenger is

$$X_T = X + X' + D'_J$$

where X_T is assumed to be constant. If the J^{th} dimer is subject to down-regulation, we assume that when the dimer complexed with messenger is removed, the messenger returns to the pool of free messenger. This is accomplished in the equations by a loss term $-\lambda D'_J$ in the equation for D'_J, a contribution of $2\lambda D'_J$ to the equation for R_I, and no change in the conservation law for messenger.

In the programs kinetic_pr_2 adn kinetic_pr_3, the equations involving messenger are written in terms of the nondimensional variables $x = X/X_T$, $x' = X'/X_T$, and $d'_J = D'_J/R_T$, and nondimensional parameters R_T/X_T and $\tilde{k}_{+x} = k_{+x}\tilde{X}_T$, where $\tilde{X}_T$ is the solution concentration of the messenger, in M. For kinetic_pr_2, the equations are

$$\begin{aligned}
\frac{dx}{dt} &= -\tilde{k}_{+x}x(R_T/X_T)d_J + (k_{-x} + 2k_{-2})(R_T/X_T)d'_J + \mu x' \\
\frac{dx'}{dt} &= k_{x'}(R_T/X_T)d'_J - \mu x' \\
\frac{dd'_J}{dt} &= \tilde{k}_{+x}xd_J - (k_{-x} + k_{x'} + 2k_{-2})d'_J
\end{aligned}$$

The equation for d_J is modified from Section 4.2 by adding $-\tilde{k}_{+x}xd_J + (k_{-x} + k_{x'})d'_J$. The nondimensional dimer concentration $d = D/R_T = \sum_{i=1}^{N} d_i + d'_J$. The conservation law for the soluble messenger is

$$1 = x + x' + (R_T/X_T)d'_J$$

If the J^{th} dimer is subject to down-regulation, an additional loss term $-\lambda d'_J$ appears in the equation for d'_J, a gain of $2\lambda d'_J$ appears in the equation for r_I, and there is no change in the conservation law for messenger.

A.4. Initiating enzyme

In the kinetic proofreading models we have described, receptor dimers undergo successive reversible modifications. If a modification represents association with an enzyme, we have effectively assumed the enzyme is in excess, so that the association rate is a constant multiple of the concentration of the dimer. In the program kinetic_pr_3, we assume that a dimer must associate with an initiating enzyme before further modifications can take place. The example that motivates this extension of the model is the family of MIRR (multisubunit immune

recognition receptors), interacting with Src family kinases to initiate a signaling cascade. Therefore, we assume that the enzyme is membrane-associated. We allow for the possibility that the initiating enzyme is in limited supply. Therefore, we treat the interaction explicitly as a bimolecular reaction and add to the model a conservation law for the enzyme. We keep track of two classes of dimers. Dimers without associated enzyme have concentrations denoted D_i and dimers associated with enzyme have concentrations D_i^* (both in number per cell, where i indicates the state of modification, $i = 1, \cdots, N$). If the J^{th} dimer mediates the activation of messenger, we have two additional dimer states complexed with the messenger, with concentrations (in number per cell) D'_J (if the dimer is not associated with the initiating enzyme) and D'^*_J (if the dimer is associated with the enzyme).

Other new definitions of concentrations and rate constants are:

E_T = total number of molecules per cell of the initiating enzyme
E = number of free enzyme molecules per cell
$e = E/E_T$
k_{+E} = rate constant for the association of the enzyme with a receptor dimer (cm^2/s)
$\tilde{E}_T$ = surface concentration of enzyme (receptors/cm^2)
$\tilde{k}_{+E} = k_{+E}\tilde{E}_T$ (s^{-1})
k_{-E} = rate constant for dissociation of an enzyme/dimer complex (s^{-1})
$D = \sum_{i=1}^{N} (D_i + D_i^*) + D'_J + D'^*_J$
$d = D/R_T$
$d_i^* = D_i^*/R_T$
$d_i'^* = D_i'^*/R_T$

The conservation law for the enzyme, in dimensional terms, is

$$E_T = E + \sum_{i=1}^{N} D_i^* + D'^*_J$$

and in nondimensional form

$$1 = e + (\sum_{i=1}^{N} d_i^* + d'^*_J)(R_T/E_T)$$

An additional initial condition for the system of differential equations, written in terms of nondimensional concentrations, is

$$e(0) = 1$$

If there is no internalization and no reaction of a dimer with a messenger, then the differential equations, written in terms of nondimensional concentrations of receptor states, are

$$\frac{db}{dt} = 2k_{+1}Lr + 2k_{-2}d - (k_{-1} + \tilde{k}_{+2}r)b$$
$$\frac{db'}{dt} = k_{+m}L'r - k_{-m}b'$$
$$\frac{dd_1}{dt} = \tilde{k}_{+2}br + k_{-E}d_1^* + k_{-p2}\sum_{i=2}^{N} d_i - (2k_{-2} + \tilde{k}_{+E}e)d_1$$
$$\frac{dd_i}{dt} = k_{-E}d_i^* - (2k_{-2} + \tilde{k}_{+E}e + k_{-p2})d_i \qquad 2 \le i \le N$$
$$\frac{dd_1^*}{dt} = \tilde{k}_{+E}ed_1 + k_{-p}d_2^* - (2k_{-2} + k_{-E} + k_{+p})d_1^*$$
$$\frac{dd_i^*}{dt} = \tilde{k}_{+E}ed_i + k_{+p}d_{i-1}^* + k_{-p}d_{i+1}^* - (2k_{-2} + k_{-E} + k_{+p} + k_{-p})d_i^* \qquad 2 \le i \le N-1$$
$$\frac{dd_N^*}{dt} = k_{+E}ed_N + k_{+p}d_{N-1}^* - (2k_{-2} + k_{-E} + k_{-p})d_N^*$$

If the J^{th} dimer activates an intracellular messenger, we assume that either of the forms, with or without enzyme, can form a complex with the messenger and activate it. The equations for states involving messenger are

$$\frac{dx}{dt} = -\tilde{k}_{+x}x(R_T/X_T)(d_J + d_J^*) + (k_{-x} + 2k_{-2})(R_T/X_T)(d'_J + d'^*_J) + \mu x'$$
$$\frac{dx'}{dt} = k_{x'}(R_T/X_T)(d'_J + d'^*_J) - \mu x'$$
$$\frac{dd'_J}{dt} = \tilde{k}_{+x}xd_J + k_{-E}d'^*_J - (k_{-x} + k_{x'} + 2k_{-2} + k_{-p2} + \tilde{k}_{+E}e)d'_J$$
$$\frac{dd'^*_J}{dt} = \tilde{k}_{+x}xd_J^* + \tilde{k}_{+E}ed'_J - (k_{-x} + k_{x'} + 2k_{-2} + k_{-E})d'^*_J$$

The equation for d_J is modified by adding $-\tilde{k}_{+x}xd_J + (k_{-x} + k_{x'})d'_J$. The equation for d_J^* is modified by adding $-\tilde{k}_{+x}xd_J^* + (k_{-x} + k_{x'})d'^*_J$.

If dimers in states I and above are subject to down regulation, we assume that both forms, with and without enzyme, are removed with rate constant λ, and that any associated enzyme is not removed from the system but returns to the pool of free enzyme. As described in the context of messenger (Section A.3), this requires additional loss terms for all dimers subject to internalization, including those associated with enzyme and/or messenger; a gain in the compartment of receptors that have been removed in the down regulation process; and no change in the conservation law for enzyme.